5-2016

Expression Patterns of the Major Vault Protein (MVP) and Cellular Vault Particles in Aquatic Animal Models

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EXPRESSION PATTERNS OF THE MAJOR VAULT PROTEIN (MVP) AND CELLULAR VAULT PARTICLES IN AQUATIC ANIMAL MODELS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

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by
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May 2016

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ABSTRACT

Cellular vaults are ubiquitous 13 mega Da multi-subunit structures that may have a role in nucleo-cytoplasmic transport. Seventy percent of the vault's mass consists of a ≈100 kDa protein, the major vault protein (MVP). Elevated MVP was first recognized as lung resistance protein (LRP) because metastatic lymphoid tumor cells in the lung over-expressed this protein following acquired resistance to traditional chemotherapy. Previous work in our lab screened a cDNA library constructed from channel catfish monocytes (42TA cells), whereby MVP was sequenced and found to be highly conserved compared to other vertebrates. This sequence was recently expressed as a recombinant protein for generating a panel of specific monoclonal antibodies (mAbs), resulting in hybridoma 3F9 secreting a mAb that recognizes MVP in all fish examined to date, as well as mammalian cells. Using immunohistochemical (IHC) approaches, mAb 3F9 staining indicates that MVP, and presumably intact vaults, is highly expressed in epithelial cells, cells of the immune system, and endothelial cells in various organs. Quantitative RT-PCR primers for channel catfish MVP were designed and used to show that gene expression can be easily detected and quantified in the well-characterized channel catfish ovarian (CCO) cell line following treatment with different classes of pharmacological agents. Using mAb 3F9, we show that MVP protein levels, and presumably intact vaults, reflect high levels of MVP gene expression in catfish. In another study, we examined MVP protein expression in tissues from Atlantic killifish collected at the Atlantic Wood (AW) site on the southern branch of the Elizabeth River of VA – a US-EPA Superfund site. Killifish from the AW site are adapted to high

concentrations of PAHs, display a PAH-resistant phenotype, and have a high incidence of liver lesions and neoplasia. MVP expression is highly variable in AW fish compared to a relatively clean reference site, and many advanced neoplastic liver lesions in AW killifish show high expression, and even nuclear location in the most advanced lesions. The true function of cellular vaults remains unknown, but the results of this study support a role in acquired resistance to intracellular toxic compounds and/or metabolites, and may be a shuttle between the cytoplasm and nucleus.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Charles D. Rice for his help and guidance throughout the course of my research. I would like to thank my committee members, Drs. Yanzhang (Charlie) Wei and Thomas R. Scott for your suggestions and assistance in my projects. I would also like to thank my lab members for their unwavering support. I appreciate all that you have done for me since the start of this journey.

Thank you to my friends and family who have continued to believe in me as I pursue my goals. Your encouragement has helped me to get to where I am today.

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CHAPTER ONE

LITERATURE REVIEW

CELLULAR VAULT PARTICLES

In 1986, Drs. Nancy Kedersha and Leonard Rome at University of California Los Angeles noticed small ovoid bodies contained within the coated vesicles of rat livers. They decided to isolate these structures using sucrose density gradients and agarose gel electrophoresis. The bodies were negatively stained with uranyl acetate and viewed under electron microscopy. Each hollow barrel-like structure is comprised of two cupped halves attached at their open ends with multiple arches that resembled cathedral vaults in radial symmetry (Kedersha et al., 1991; Kedersha and Rome, 1986a). The partially cylindrical structure also has an invaginated waist and natural flexibility (Kong et al., 1999). Measuring 35 x 65 nm with a molecular weight of 13 MDa, the particles now known as cellular vaults are considerably larger than ribosomes and are the largest known subcellular ribonucleoprotein bodies (Esfandiary et al., 2009). A few years after discovery in the rat livers, cellular vaults were isolated in various eukaryotes, including mammals, amphibians, aves, and even the lower eukaryote *Dictyostelium discoideum.* Across all species tested to date, vault particles are highly conserved in dimension, morphology, and protein composition (Kedersha et al., 1990a). Vaults are consistently found in their highest numbers in macrophages and epithelial cells along the digestive tract (Kedersha and Rome, 1990b).

Vaults are slightly smaller than most coated vesicles and differences between the two have been analyzed. Coated vesicles contain clathrin, a protein with triskelion shape

that forms a protective lattice around the vesicle. Coated vesicle preparations containing vaults were treated with urea to remove clathrin and the vaults remained intact. This suggested the need for further research on the extreme stability of cellular vaults (Kedersha and Rome, 1986a). Cellular vaults have been found to be most stable between pH 6-8 and below 40°C. With extreme pH and temperatures, cellular vaults morph into ten different conformational states, including half vaults, aggregates, molten globule-like structures, and open flower-like structures. These variations likely play a role in the vault function (Esfandiary et al., 2009; Kedersha et al., 1991).

While the true function of cellular vaults is currently unknown, the structure of vaults is very similar to that of nuclear pore complexes (NPCs). The outer diameter of an NPC is approximately 120 nm, just slightly larger than that of the flower-like open vault structures of 100 nm. Also the NPC polypeptides are nearly identical in mass to the major polypeptides of the vault. This suggests cellular vaults could be precursor components of NPCs (Kedersha et al., 1991). In addition, the central plug of the NPC is an ovoid structure with a mass of 13 MDa (Reichelt et al., 1990), which is identical to that of a vault (Kedersha et al., 1991). There is significant evidence that the central plug and NPC as a whole both have the same symmetry as cellular vaults, with bilateral symmetry and each half holding eightfold radial symmetry (Akey, 1990). Using immunoblotting and immunofluorescence, Chugani, et al. (1993) discovered that while vaults are contained mostly within the cytoplasm, they also interact with the nuclear envelope and NPCs. There is not enough evidence to date to conclude whether or not cellular vault particles actually form the central plug itself, but vaults are likely involved in nucleocytoplasmic transport.

In addition to interacting with the nuclear membrane, a subpopulation of vaults is associated with microtubules. These vaults also remain associated with tubulin dimers and oligomers when microtubules are disassembled. Nearly always, cellular vaults bind to microtubules via one of their vault caps, perpendicular to the long axis of the tubule. Each micron of a microtubule holds 5 to 6 vault particles. The binding and release of cellular vaults to microtubules suggests a mode of transportation for cellular vaults throughout the cytosol (Eichenmuller et al., 2003).

MAJOR VAULT PROTEIN

Cellular vaults are comprised of several different components in varying quantities and importance. The three dominant proteins have molecular weights of 240 kDa, 193 kDa, 100 kDa. These were found to be telomerase protein component 1 (TEP-1), vault poly-(ADP)-ribose polymerase (vPARP), and major vault protein (MVP), respectively (Kedersha and Rome, 1986a; Kickhoefer et al., 1999c; Kickhoefer et al., 1999d). The final notable component is vault ribonucleic acids (vRNA) with a molecular weight of 37 kDa (Kickhoefer et al., 1993).

About 74.6% of the cellular vaults are comprised of the approximately 100 kDa protein (Kedersha et al., 1991). Being the main protein with the most direct function, it is now referred to as the major vault protein (MVP), ranging in size from 95-104 kDa, depending on the species (Kedersha et al., 1990a). The amino acid sequence of 110 kDa lung resistance-related protein (LRP) shows 87.7% identity with the 104 kDa rat liver major vault protein. Therefore, they are now classified as the same protein and more commonly called MVP (Scheffer et al., 1995a).

The petals of the open flower formation each contain six molecules of p104 (MVP) and give the vault its barrel-shaped structure (Kedersha et al., 1991). MVP molecules interact with each other via their coiled coil domain in their C-terminal halves. These MVP-MVP subunits are the basis of vault assembly (van Zon et al., 2002). When this junction is cleaved, the vaults either collapse on their sides or open into the flower formation. The same study concludes that it is likely that p210, p192, and p54 make up the central ring and extension to the petals (Kedersha et al., 1991).

MULTIDRUG RESISTANCE

Multidrug resistance (MDR) is the main cause of failure of cancer treatments utilizing chemotherapy (Lehnert, 1996). Overexpression of transporter molecules, which act as drug efflux pumps, can cause a decrease in intracellular drug accumulation and enhance MDR. Two common transporter molecules that produce this effect include P-glycoprotein (Pgp) and multidrug resistance-associated protein (MRP) from the ATP-Binding Cassette (ABC) superfamily (Izquierdo et al., 1996; Schroeijers et al., 2001a). The sequestering of drug into exocytotic vesicles has been recently called into question as it relates to the suspected function of cellular vaults. (Chugani et al., 1993b; Scheffer et al., 1995a) Cellular vaults are similarly sized to the central plug of the NPC and many studies have supported that these particles play a role in nucleo-cytoplasmic vesicular transport of compounds such at cytostatic and cytotoxic drugs (Abbondanza et al., 1998; Chugani et al., 1993b; Herrmann et al., 1999; Herrmann et al., 1996; Kong et al., 1999). Cellular vaults are not classified as being a part of the ABC superfamily, making them of particular interest.

An overexpression of LRP/MVP, vRNA, and vaults has been directly linked to multidrug resistance in diverse cancer cell lines. (Kickhoefer et al., 1998; Moran et al., 1997; Schroeijers et al., 2001a). In MDR cell lines, the expression of LRP/MVP and P-glycoprotein are exclusive, meaning if Pgp is overexpressed, LRP/MVP is not and vice versa (Moran et al., 1997; Scheper et al., 1993a). In contrast, MDR cell lines with overexpression of LRP/MVP tend to also have high levels of MRP (Flens et al., 1994; Scheper et al., 1993a). Specifically, MVP has been analyzed in its resistance to chemotherapy drugs including doxorubicin, vincristine, etoposide, and paclitaxel, as well as the topical antibiotic gramicidin D. Doxorubicin has also been traced to travel from nucleus to cytoplasm with the help of LRP (Kitazono et al., 1999b). Results from some clinical studies showed that LRP/MVP expression is a strong independent prognostic factor for resistance to chemotherapy and/or poor outcome (Izquierdo et al., 1998). The LRP gene may mediate drug resistance through a transport process due to the fact that it is located close to the multidrug resistance-associated proteins (MRP) genes on chromosome 16 (Scheffer et al., 1995a).

Polyclonal and monoclonal antibodies that recognize LRP/MVP are critical for the analysis of vault-associated MDR. They are utilized in the characterization of the MVP protein (Scheffer et al., 1995a; Scheper et al., 1993a) as well as in translocation studies of chemotherapy drugs (Kitazono et al., 1999b). Antibodies have been used n immunohisto- and cytochemical evaluations to analyze the MVP distribution among tissues and tumors as well as its subcellular localization (Scheper et al., 1993a; Schroeijers et al., 2001a).

AQUATIC MODELS AT ATLANTIC WOOD

Countless human disease research studies utilize aquatic animal models largely in part due to the convenience of quick growth and low regulation of fish. With over 20,000 species, fish are the most diverse and oldest group of vertebrates (Hickman CPJ, 1984). Even though most vertebrate processes are highly conserved, mammals and bony fish diverged about 400 million years ago, and therefore observations made in fish must be carefully analyzed before being applied to humans. Models of carcinogenesis, mutagenesis, and toxicology commonly use aquatic models, especially estuarine species, as they are likely to have high exposure to industrial contaminants and pathogens.

The Elizabeth River of the Chesapeake Bay in Virginia is a highly contaminated subestuary due to several wood treatment industries along its banks. A common toxicant dumped into the river is creosote, a mixture of chemicals predominantly consisting of unsubstituted polycyclic aromatic hydrocarbons (PAHs), as well as some heterocyclic and phenolic PAHs (Clark et al., 2013; Fang et al., 2014). The Atlantic Wood (AW) Industries EPA Superfund site holds PAH concentrations ranging from 100 to 500 μg/g (Mulvey et al., 2002).

Fundulus heteroclitus, also known as the Atlantic killifish or mummichog, is the most abundant intertidal fish species in these estuaries. Although widespread, killifish have small migration areas, staying in the same area for their lifetime. This characteristic makes them ideal for studying the effects of PAH contaminants at the AW site (Lotrich, 1975; Skinner et al., 2005). These killifish have developed a significant resistance to the cardiotoxicity and teratogenic effects of the PAH-contaminants, which is linked to the down regulation of the aryl hydrocarbon receptor (AhR) pathway (Billiard et al., 2004;

Meyer and Di Giulio, 2002). They have also been able to thrive despite other contaminants such as polychlorinated biphenyls (PCBs) (Nacci et al., 1999; Nacci et al., 2002), heavy metals (Weis, 2002), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) (Prince and Cooper, 1995a, b).

Similar studies however have shown that fish exposed to these contaminants have liver lesions and altered enzymatic activities. As compared to non-polluted sites, killifish at the AW site have increased glutathione reductase and glutathione peroxidase activities (Bacanskas et al., 2004). Cytochrome P450 1A (CYP1A) of the AhR pathway is a critical component to the breakdown of many harmful contaminants. Interestingly, in highly contaminated sites such as Atlantic Wood, CYP1A is reduced (Meyer and Di Giulio, 2002).

CHAPTER TWO

EXPRESSION OF THE MAJOR VAULT PROTEIN (MVP) AND VAULT PARTICLES IN HEALTHY AND DISEASED FISH CELLS AND TISSUES

INTRODUCTION

Cellular vault particles are large (13 MDa) multi-unit ribonucleoprotein complexes (65 x 45 nM) ubiquitously expressed in the cytosol and nucleus of most eukaryotic cells (Kedersha and Rome, 1990a; Kedersha and Rome, 1986b). The barrel-shaped vault complex is composed of ≈ 240, ≈ 193, and ≈ 100 kDa proteins, and one small non-coding vRNA. Nearly 70% of the vault mass is comprised of the ≈ 100 kDa protein, the major vault protein (MVP) (Mossink et al., 2003; van Zon et al., 2003). The 193 kDa component contains poly (ADP-ribose) polymerase activity, and now referred to as the vault PARP (vPARP) (Kickhoefer et al., 1999a), while the 240 kDa component was shown to be identical to a previously described component of the telomerase complex, the telomerase-associated protein-1 (TEP1) (Kickhoefer et al., 1999b; van Zon et al., 2001). The small non-translated RNA molecules of the vault complex vary between animal phyla, and contain different structures (Stadler et al., 2009), with hints that vRNA may code for miRNAs, including *mir-733* or others that regulate drug metabolism and acquired resistance to drugs (Gopinath et al., 2010; Persson et al., 2009).

Vaults may be a component of the nuclear pore complex (NPC) known as the central plug or NPC transporter (Chugani et al., 1993a). The mass of this central plug is approximately 13 kDa, the same as for vaults, and both have the same multi-unit configuration of two halves with an eight-fold symmetry. Thus, vaults may be involved

in the transport of materials between the nucleus and the cytoplasm (Slesina et al., 2006; Slesina et al., 2005)

Though an absolute function of cellular vaults has not been determined, assembly of the vault is absolutely dependent on the expression of the MVP component, and therefore MVP expression is considered a marker for vault expression (van Zon et al., 2003). The MVP, and therefore vaults, seem to be most abundant in macrophages, dendritic cells, and epithelial cells (Berger et al., 2009; Schroeijers et al., 2001b; Steiner et al., 2006b), however their true function(s) is unknown. MVP is over-expressed in ovarian carcinoma, acute myeloid leukemia, and non-small cell lung carcinoma, and was originally named lung resistance protein (LRP) (Kitazono et al., 1999a; Ohno et al., 2001; Scheper et al., 1993b). It is often over-expressed in P-glycoprotein (MDR1)-negative multidrug resistance cancer cell lines (Scheper et al., 1993; Scheffer et al., 1995), in some multidrug resistance-associated protein (MRP1)-negative lines, and is involved in resistance to doxorubicin, vincristine, etoposide, and taxol (Kitazono et al., 1999a). Furthermore, chronic exposure to low levels of the polycyclic aromatic hydrocarbon benzo-*a*-pyrene (BAP) upregulates MVP expression in KB-3-1 epidermal pharyngeal carcinoma cells, both at the message and protein level (Cheng et al., 2000). Expression of MVP in their system was associated with enhanced resistance to the cytotoxic agent doxorubicin.

Clearly, most of the literature on MVP, and thus cellular vaults, has focused on mammalian cells, with particular attention to cellular signaling (Ben et al., 2013; Steiner et al., 2006b) and drug resistance, or a role in tumorigenesis (Silva et al., 2007). However, expression patters of MVP, and thus cellular vaults, on lower vertebrates, and

especially aquatic animals commonly used as biomedical models for human diseases, is lacking. To date, MVP has been identified and examined in Dictyostellum, frogs, sea urchins, birds, rays, and all mammalian species examined, but not flies (Kedersha et al., 1990b).

This particular study herein describes the generation of a monoclonal antibody exhibiting cross reactivity with a wide variety of teleostean fishes, and even mammalian MVP. The expression patterns of MVP protein under normal and pathological conditions is examined, as well as both mRNA and protein in a cell line following exposure to potent pharmacological inducers of cell signaling. A former student in the Rich lab produced a cDNA library from the channel catfish monocyte cell line 42TA, and screened the library to reveal a 900 base pair sequence matching with high homology to MVP of rat, human, a ray, and to some degree a mussel, and deposited in Genebank under accession # AF255664. This sequence was expressed in a pQE31 expression plasmid for expressing a recombinant MVP protein.

The development of a monoclonal antibody for fish MVP provides an excellent opportunity to examine the possible role of this protein, or at least a correlation of MVP, with liver lesions in a population of Atlantic killifish, *Fundulus heteroclitus*, known to have acquired resistance to the acute toxicity of polyaromatic hydrocarbons (PAHs) at the Atlantic Wood Superfund Site on the Southern branch of the Elizabeth River, VA, near Portsmouth. These fish exhibit a high incidence of liver lesions, including hepatic neoplasia, even though as embryos and adults are they are resistant to both acute and developmental toxicity of PAHs compared to killifish at a well-characterized reference site. The exact mechanism(s) associated with the resistance phenotype are still unknown,

but to date a possible role of MVP, and thus vaults, has not been examined in the Atlantic Wood killifish population.

MATERIALS AND METHODS

Monoclonal Antibody Generation and Characterization

Six-week old female Balb/c mice were obtained from a commercial source and houses at the Godley-Snell Animal Facility at Clemson University under IACUC approved protocols. Mice were given a sub-cutaneous (s.c.) injection of 100 µg recombinant channel catfish MVP in 0.9% saline containing TiterMax Gold adjuvant on Day 1. Fourteen days later mice received a second s.c. immunization using Freund's incomplete adjuvant. Subsequent boosters at 21 day intervals were given in saline only via intraperitoneal injections. Five days after the last booster immunization, mice were sacrificed using slow lethal CO_2 hypoxia, and their spleens removed using aseptic methods. Procedures for fusion with Sp02 myelomas, and for screening and cloning of the resulting hybridomas have been described elsewhere (Rice et al., 1998). Hybridomas were typically grown in Dulbecco's Modified Eagle Medium (DMEM, Cellgro) supplemented with iron-supplemented fetal calf serum (FCS), 20 mM HEPES, 10 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 110 µg/ml sodium pyruvate, 1% non-essential amino acids (100x stock), 4.5 g/L glucose, 10 µg/ml gentamycin and 5 µg/ml nystatin.

At confluence, the supernatants were collected by centrifugation and treated with 0.5 % NaN_3 and stored at 4^0 C. Isotyping the antibodies within hybridoma supernatants was carried out using Pierce™ Rapid Antibody Isotyping Kits for mouse (ThermoFisher). To obtain purified antibody, hybridomas were grown in the same supplemented media, but 10% FBS containing super low bovine IgG (Thermo Fisher). The supernatants were

then mixed 1:1 with a saturated NH_4SO_4 solution in DI water, and allowed to cool at 4^0 C overnight, at which time the mixture was centrifuged at 3000 times gravity to collect the pelleted precipitated proteins. The pellet was resuspended in PBS at one-tenth the volume of original supernatant, then dialyzed extensively against PBS at 4^0 C to remove the NH_4SO_4. The resulting product was then removed from dialysis bagging and passed through a protein A/G column (Thermo Fisher) to isolate IgG immunoglobulins following the manufacturer's instructions (Thermo Fisher), which were then stored at 40 C with 0.5% NaN_3.

Determining reactivity of anti-catfish MVP antibody against rMVP, native MVP, and MVP with other species of fish and rodent MVP.

To determine the reactivity of anti-catfish MVP antibody with rMVP and native proteins, 1 µg of rMVP or 20 µg of 42TA catfish monocyte cell lysates were subjected to SDS-PAGE and transferred to PVDF membranes as described above. After blocking with 10% FBS in PBS, the membranes were probed with confluent hybridoma supernatants for 1 hr at room temperature, washed 3x with PBS-Tween 20, then probed again with goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (1:1500 in PBS) (ThermoFisher) for 1 hr at room temperature. Following three washings in PBS-T20, alkaline phosphatase activity was visualized with BCIP/NBT. To evaluate the reactivity of anti-rMVP antibodies with MVP from representative teleostean fishes commonly used as aquatic animal models, in hand tissue lysates from rainbow trout (Oncorhynchus mykiss), zebrafish (Danio rerio), and Atlantic killfish (Fundulus

heteroclitus) used in previous studies (Marsh and Rice, 2010) were also subjected to SDS-PAGE and immunoblotting steps.

Cellular and sub-cellular localization of MVP in fish tissues

Rainbow trout tissues were generously provided by the SC Department of Natural Resources at the Walhalla Fish Hatchery, Walhalla, SC USA. Fish were lethally sedated in MS-222 buffered with sodium bicarbonate, and livers, gills, anterior kidney, posterior kidney, spleens, and intestines were quickly removed onsite and placed in 10% buffered formalin containing zinc salts (Z-Fix, Anatech, Ltd, Battle Creek, MI, USA). After 72 hr fixation, the Z-fix solution was removed and replaced with 70% ETOH until processing for paraffin embedding.

Tissues were embedded and processed for sectioning and mounting at the College of Veterinary Medicine, Mississippi State University, core histology facility. Five μm sections were cut on a rotary microtome from individual tissues, and a representative section of each tissue was stained for H&E, and other sections were provided for immunohistochemistry (IHC). Slides were heated in Tris-EDTA buffer, pH 9, by microwave on 100% power for 5 minutes followed by cooling for 5 min, followed by a final 5 min 100% power, and a final 20 min rest in the container. Tissues on slides were encircled with a Liquid Blocker Super mini pen to separate tissue slices, then the appropriate antibody, as hybridoma supernatant diluted 1:20 in PBS, was added and incubated overnight at 4°C. Each slide with tissue slices contained one slice receiving secondary antibody only as a control. Additional screening assays included isotype controls for the mAbs. The slides were then washed and endogenous peroxidases

quenched with 3% hydrogen peroxide, and an avidin-biotin blocking step (Vector Labs) was included. Tissues were further processed using a horse-anti-mouse IgG Vectastain ABC-Ultra kit as directed by the kit. Antibody labeling was detected with Nova Red staining, and counter-stained with hematoxylin (Vector Labs).

To visualize and localize intact cellular vault particles, channel catfish lymphoid tissues were collected and prepared for single cell suspension as previously described using percoll gradients and centrifugation (Marsh and Rice, 2010). Resulting phagocytes (mostly neutrophils and macrophages) were allowed to adhere to pre-cleaned and poly-L-lysine treated glass cover slips for 3 hr, then probed with mAb 3F9 as a 1:4 dilutions of hybridoma supernatant in PBS, and allowed to incubate at room temperature for 2 hr with gentle shaking. The slips were washed 3x in PBS-T20 for 10 min each wash, then covered with goat-anti-mouse IgG-alexafluor 488 (Life Technologies, 1:250 in PBS) for another 2 hr at room temperature under gentle rocking. Finally, the slips containing cells were washed 3x with PBS-TW and examined by epifluoresence. Images of these cells were captured using the LASX analysis software on a Leica TCS SPE scope.

The effects of pharmacological and toxicology treatment on MVP expression in vitro with the channel catfish ovarian cell line CCO.

Channel catfish ovarian cells (CCO) were generously provided by Dr. Larry Hanson, College of Veterinary Medicine, Mississippi State University, and maintained at 30°C in the same medium as described above for hybridoma maintenance. Cells were typically grown in T-75 or T-150 Corning flasks until the time of experimental use. For MVP mRNA expression studies, CCO cells were seeded into 7-25 flasks containing 5 ml

complete media until near confluence. For MVP protein expression studies, cells were grown to near confluence in T-75 flasks containing 15 ml complete media. For intracellular localization of MVP, cells were grown to near confluence over pre-cleaned glass cover-slips in 6-well plates containing 3 ml per well of complete media. After allowing cells to adhere to bottom substrate, cells were treated with the individual treatments supplied in Table 1.

Experimental exposures were carried out in triplicate, and cells were treated for 24 hr, then pulsed again for another 24 hr, at which time the media was removed to obtain cells for further analysis. Cells were collected using 500 µl of RNAzol® (Molecular Research Center, Inc.). RNA was then isolated per the manufacturer's instructions. cDNA was synthesized using qScript cDNA Supermix (Quanta) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the SensiFAST™ SYBR® & Fluorescein kit (BioLine) according to the manufacturer's instructions, with the primer sets in Table 2. Each reaction was performed in triplicate. Plates were run and data was collected using the BioRad IQ5 detection system. Data were expressed as fold increase in expression compared to 18S house-keeping gene expression using the Pfaffl method (Pfaffl, 2001)

Cells for protein examination were trypsinized and pelleted prior to lysis in RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Pierce). The cell pellet was disrupted by gentle vortexing, and incubated on ice for 30 min, then centrifuged at 1000 ×*g* for 10 min. The overlying supernatant was removed and centrifuged again for

20 min at 14,000 ×*g*; the overlying supernatant was again removed and its protein content quantified.

Fifty micrograms of lysate protein from each sample were separated by SDS-PAGE on 7.5 % gels and transferred overnight onto PVDF membranes at 4°C. The membranes were washed 3x with 0.01 M phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBS-tw20), covered with blocking buffer (10% FCS in PBS) for 1 h and again washed 3x with PBS-tw20. Membranes were then probed for MVP using mAb 3F9 as confluent hybridoma supernatants over a 1 hr incubation period. The blots were washed 3x for 10 min with PBS-tw20, and probed for 2 h with goat-anti mouse IgG-AP (SouthernBiotech) diluted 1:1000 in PBS-tw20 containing 1% FCS. After extensive washings with PBS-tw20, alkaline phosphatase activity was determined using the substrate NBT-BCIP as a means to visualize the relative amount of specific protein. Band densities for each protein were quantified using BioRad G-17 documentation system, and the relative amount of MVP protein expression were normalized to the β-actin loading control. Data were expressed as relative expression compared to β-actin.

Treated CCO cells adhered to glass cover slips were washed with PBS-T20, then permeabilized with 0.2% triton-x in PBS over a 30 min period. Cells were then was again with PBS-T20 and blocked for 1 hr with 10% FCS in PBS. Following additional wash steps, cover slips were probed with mAb 3F9 as described above.

MVP expression patterns in liver lesions of killifish adapted to creosote at the Atlantic Wood EPA Superfund Site.

Formalin-fixed and paraffin embedded liver tissues from Atlantic killifish collected at the Atlantic Wood EPA Superfund site in VA, near Portsmouth were provided as a generous gift from Dr. Wolfgang Vogelbein (fish histopathologist) of the Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA. 5 μm slices from various livers harboring different lesions and types of tumors were provided on charged glass slides, and used to probe for MVP expression as described above for rainbow trout tissues. A diagnosis for each liver lesion type was provided by Dr. Vogelbein.

Random frozen killifish livers from the Atlantic Wood site and from a nearby reference site were also provided by Dr. Vogelbein. These tissues were homogenzized in 1 ml homogenization buffer [25 mM MOPS (pH 7.5), 1 mM EDTA, 5 mM EGTA, 20 mM Na_2MoO_4, 1 mM DDT, 10% glycerol, 0.02% NaN_3], containing 2 x of HALT protease inhibitor cocktail (Pierce) using a mini bead-beater (3110BX) with 1 ml of 1 mm glass beads (Biospec). Homogenizations were carried out in XXTuff 2 ml microvials (Biospec) at 4800 rpm over a 3 min period. Tubes were quickly placed on ice for 5 min then shaken again for 3 min, followed by another 5 min on ice, then one final 3 min shake. Tube contents were removed by pipetting and centrifuged in a clean 1.5 ml snap cap tube at 1000 x g for 3 min to pellet debris and organelles. The overlying lysate was then centrifuged at 16,000 x *g* to obtain S9 fractions for SDS-PAGE and immunoblotting. Thirty μg of sample were subjected to SDS-PAGE on 4-20% gels, then transferred to 0.45μM Immunlon (PVDF) membrane at 4^0C overnight at 35 V. Immunoblotting steps leading to visualization of MVP were the same as described above.

Table 1

Treatment	Dose	Known effects
DMSO	1 µM	Experimental control
Ethidium bromide	1.5 µg/ml	Causes DNA strand breaks
Retinoic acid	1 µM	Induces terminal differentiation
PMA + A23187	0.1 µM + 5×10^{-7} M	Stimulates the IP3 and DAG signaling cascade
Benzo-(k)-fluoranthene	1 µM	A potent agonist for the AhR
Arsenic	0.5 µM	A potent oxidative stress inducing metal

Table 1: Known cytotoxic agents administered to the CCO cells to observe MVP expression.

Table 2

Gene	Primer sequence 5’ to 3’	Anneal Temp
MVP	F: TGATTTGGTCATGCTGGGTCCTGA	60°C
	R: TAATGACGTTAGCTCGCTTGGGCT	
18S	F: GACACGGAAAGGATTGACAGA	53°C
	R: GAGTCTCGTTCGTTATCGGAATTA	

Table 2: Primer sets for MVP and 18S

RESULTS

Determining reactivity of anti-catfish MVP antibody against rMVP, native MVP, and MVP with other species of fish and rodent MVP.

Recombinant MVP (rMVP) and full length MVP were examined through SDS-PAGE and immunoblotting analysis (Figure 1). rMVP, 42TA catfish monocyte cell lystates, and killifish liver homogenates (S9 fraction) were probed with the anti-catfish MVP antibody (3F9). Recombinant MVP was expressed at 30 kDa and full length MVP was expressed at 100 kDa.

To analyze species cross reactivity, rainbow trout (*Oncorhynchus mykiss*), Atlantic killifish (*Fundulus heteroclitus*), and zebrafish (*Danio rerio*) tissue lysates as well as mouse (*Mus musculus)* macrophage cell line RAW267.4 were also subjected to SDS-PAGE and immunoblotting analysis with monoclonal antibody 3F9 (Figure 2). Monoclonal antibody 3F9 is highly reactive with all fish MVP examined to date, and works well in mammalian cells as well.

Figure 1

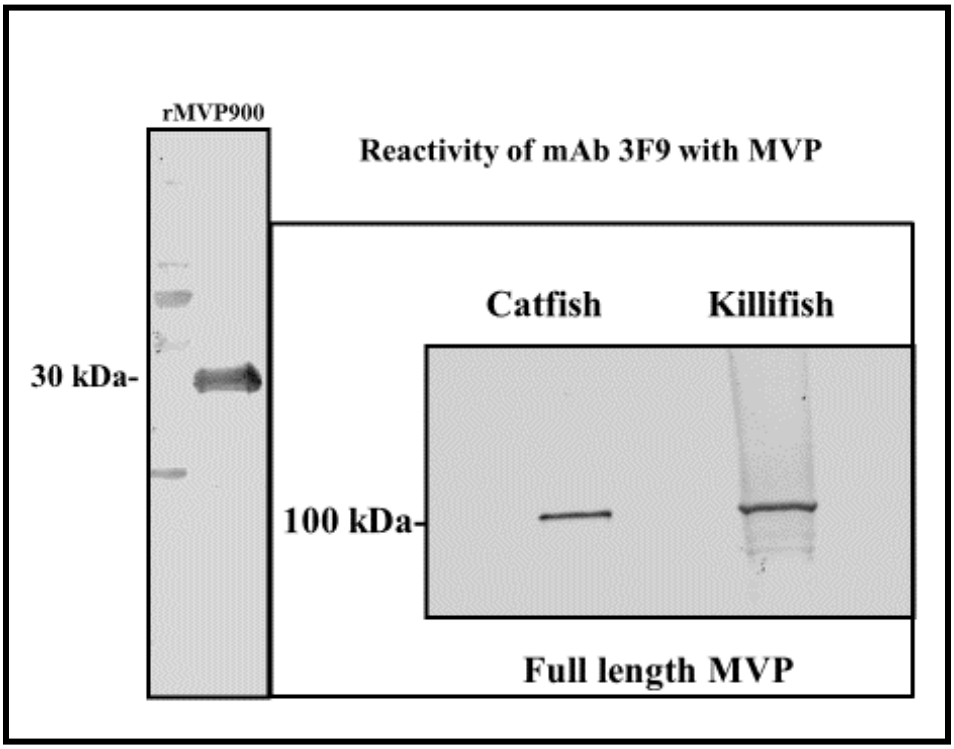


Figure 1. Western blot of reactivity of mAb 3F9 with recombinant MVP (rMVP900; left) and full length MVP in 42TA catfish monocyte cell and Atlantic killifish (*Fundulus heteroclitus*) tissue lysates (right).

Figure 2

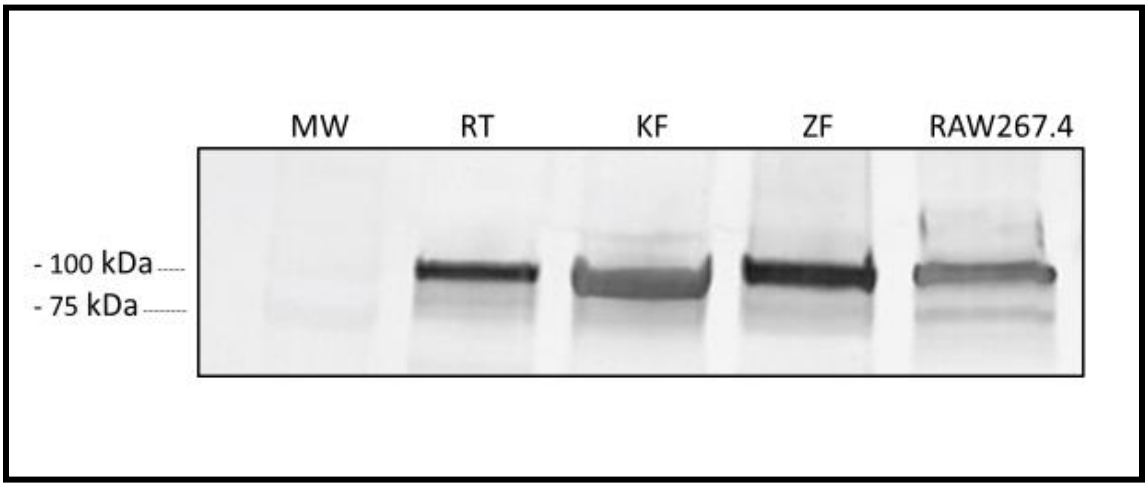


Figure 2. Species Cross Reactivity with mAb 3F9 for Major Vault Protein. Species examined by western blot included rainbow trout, killifish, zebrafish and the mouse macrophage cell line RAW267.4.

Cellular and sub-cellular localization of MVP in fish tissues

To analyze the localization of MVP within fish tissues, rainbow trout tissues (5 μm) were stained for H&E and analyzed by immunohistochemistry (IHC; Figures 3-8). In particular, a high expression of MVP identified by ImmPact NovaRed was seen in the epithelium of the gills (Figure 3), macrophages of the head kidney (Figure 4), endothelium of the intestines (Figure 5), sinusoids of the liver (Figure 6), secretory cells of the renal kidney (Figure 7), and red pulp of the spleen (Figure 8). Nuclei were counterstained with hemotoxylin.

To visualize and localize the intact cellular vault particles, channel catfish phagocytes, mostly neutrophils and macrophages, were probed with monoclonal antibody 3F9. The cells were analyzed by immunofluorescence and revealed vault particles (Alexafluor 488) concentrated in the cytoplasm (Figures 9 and 10). Note that vault numbers are lower in lymphocytes than the larger macrophages due to lower cytoplasmic space.

Figure 3

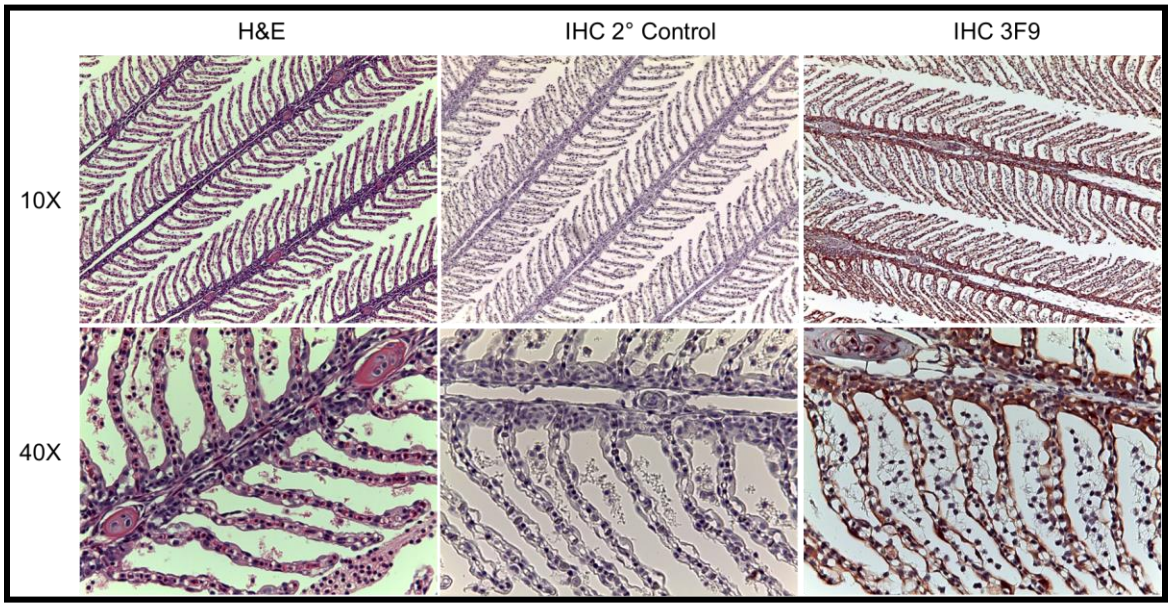


Figure 3. Rainbow trout gills. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 monoclonal antibody at 10X and 40X.

Figure 4

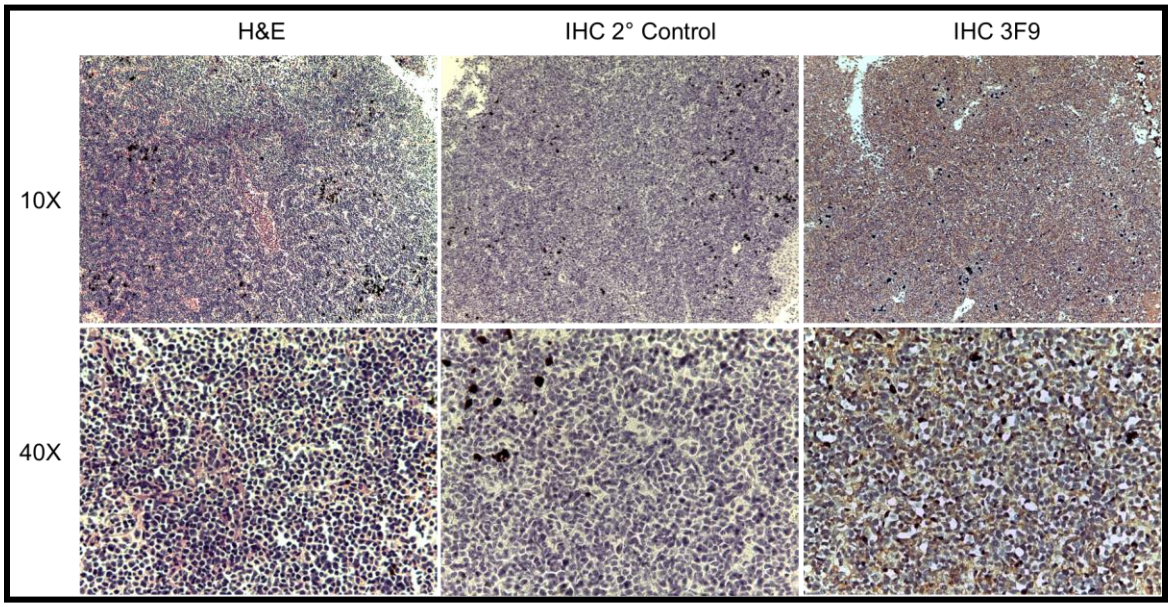


Figure 4. Rainbow trout head kidney. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 antibody at 10X and 40X.

Figure 5

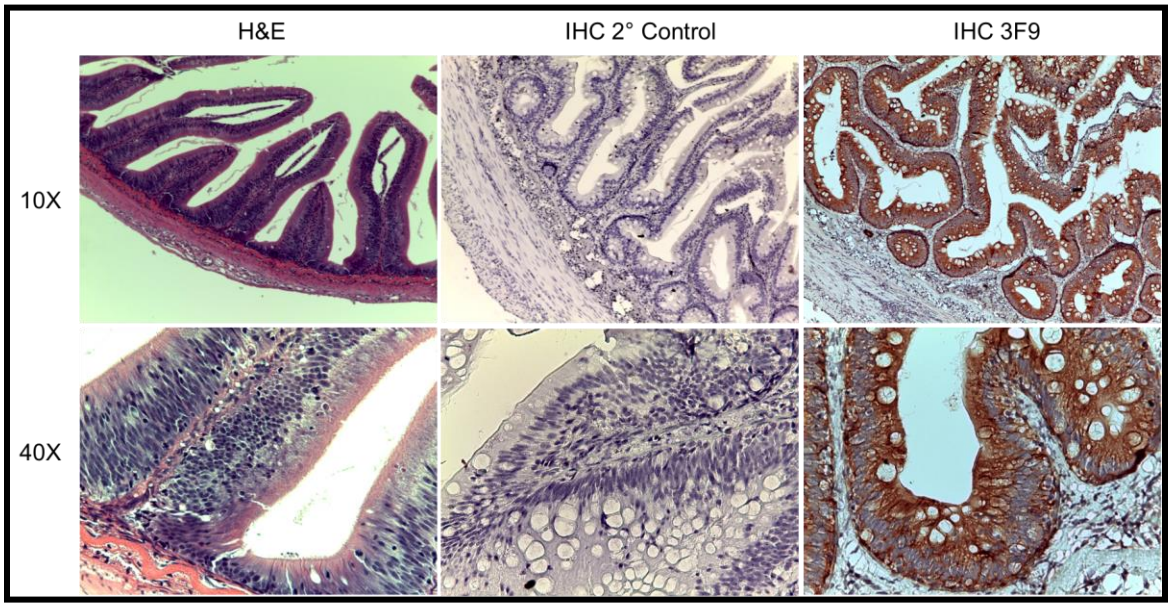


Figure 5. Rainbow trout intestines. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 antibody at 10X and 40X.

Figure 6

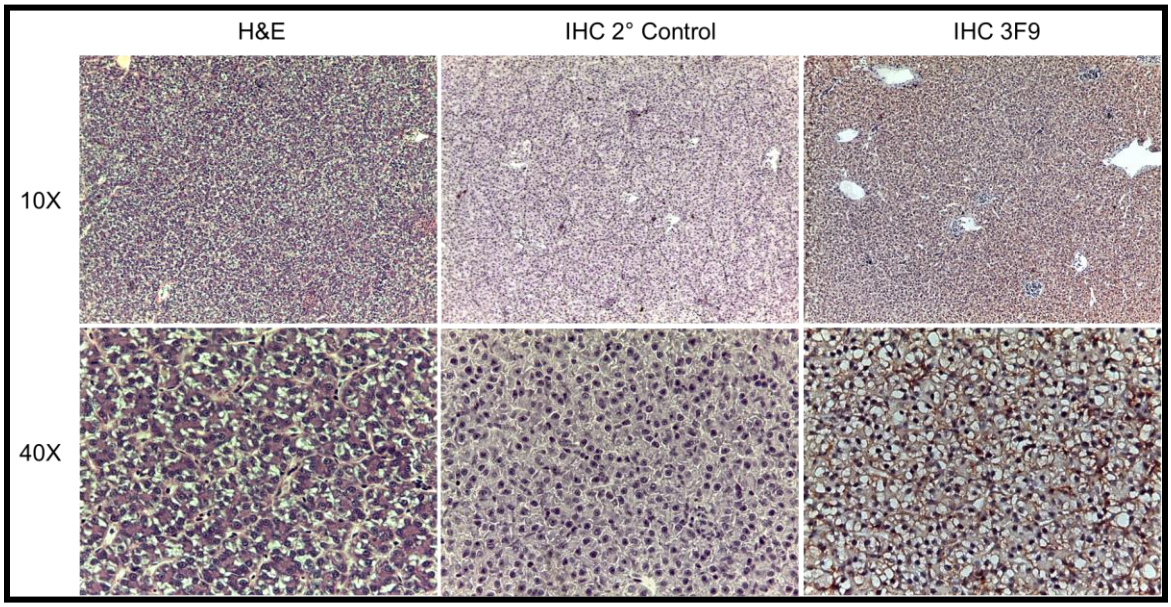


Figure 6. Rainbow trout liver. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 antibody at 10X and 40X.

Figure 7

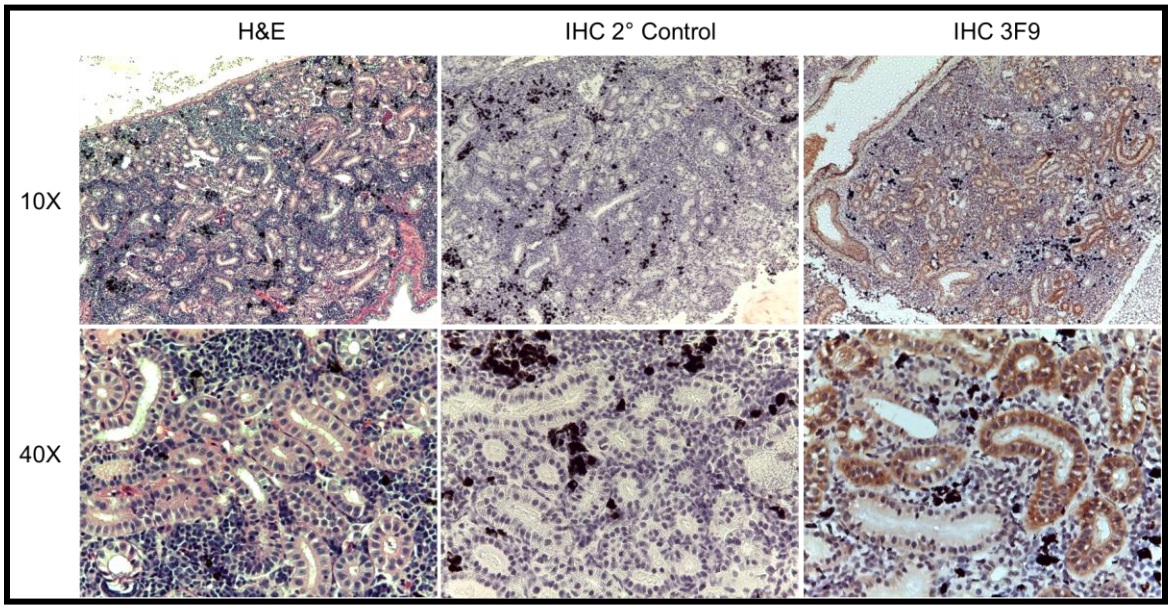


Figure 7. Rainbow trout renal kidney. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 antibody at 10X and 40X.

Figure 8

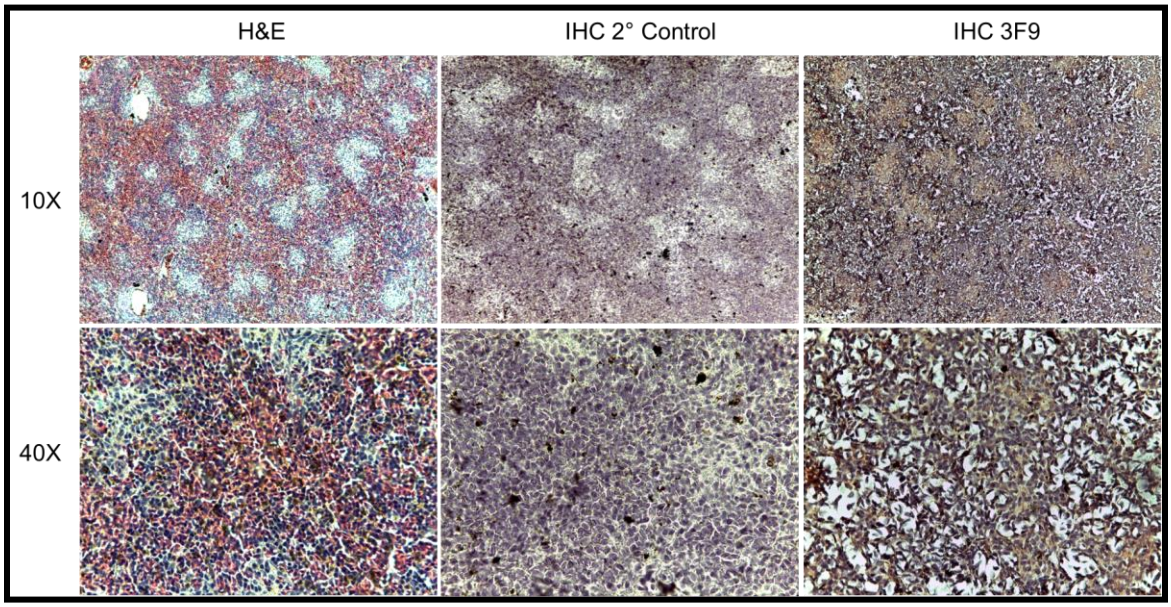
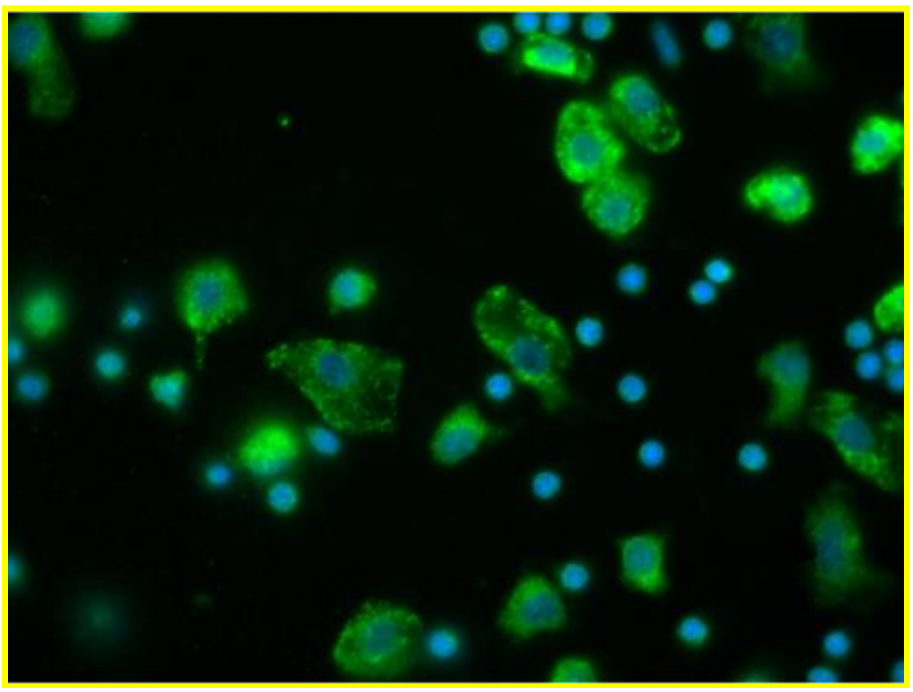


Figure 8. Rainbow trout spleen. A. Hematoxylin and eosin staining at 10X and 40X. B. Immunohistochemical staining secondary antibody control at 10X and 40X. C. Immunohistochemical staining using 3F9 antibody at 10X and 40X.

Figure 9

A



B

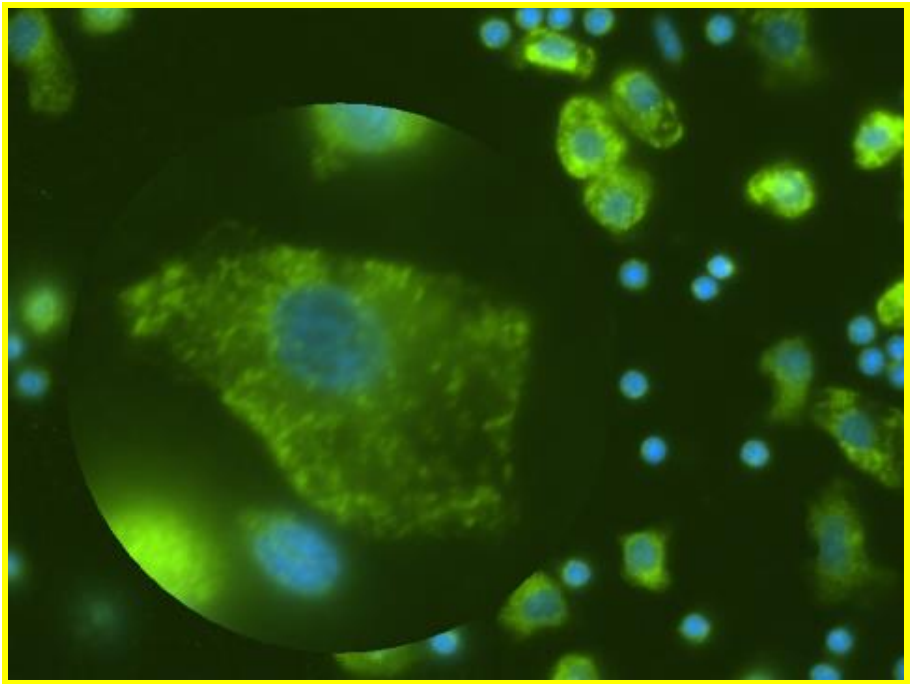


Figure 9. Immunofluorescence of MVP using IgG-alexafluor 488 and nuclear DAPI. A. Within various channel catfish phagocytes. B. Enlarged image of a macrophage.

Figure 10

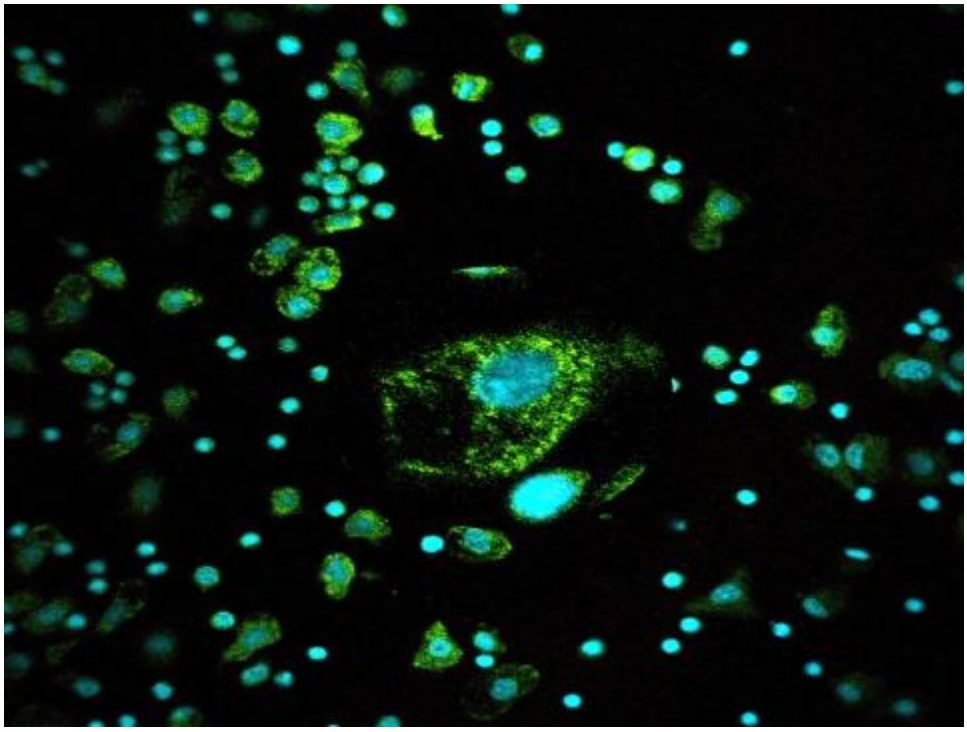


Figure 10. Confocal stacking immunofluorescence of MVP using mAb 3F9 and IgG-alexafluor 488 revealing vault particles in channel catfish phagocytes.

MVP expression patterns in liver lesions of killifish adapted to creosote at the Atlantic Wood EPA Superfund Site.

To analyze differences in MVP expressions in creosote-exposed fish directly from the Atlantic Wood superfund site, 4 females and 4 males from both Kings Creek (KC) and Atlantic Wood (AW) were collected and lysed for SDS-PAGE and immunoblotting with mAb 3F9 (Figure 11). Atlantic Wood livers revealed large variations in the expression of MVP as compared to the relatively clean control, Kings Creek site.

To determine the expression patterns of MVP in various fish tissues, Kings Creek and Atlantic Wood killifish organs were harvested, lysed, and pooled for SDS-PAGE and immunoblotting with mAb 3F9 (Figure 12). No significant variations of MVP expression were seen between the head kidneys, spleens, brains, and hearts from KC and AW. Also, there was no significant difference in the expression of MVP between the male and female samples at KC and AW.

To determine the localization of MVP and therefore cellular vaults in creosote-contaminated tissues, Atlantic Wood killifish livers were probed with mAb 3F9 through immunohistochemistry (IHC). Lesions within the frozen liver slices revealed altered cell morphology as well as high expression of MVP as compared to surrounding healthy tissue of the same specimen (Figure 13).

Figure 11

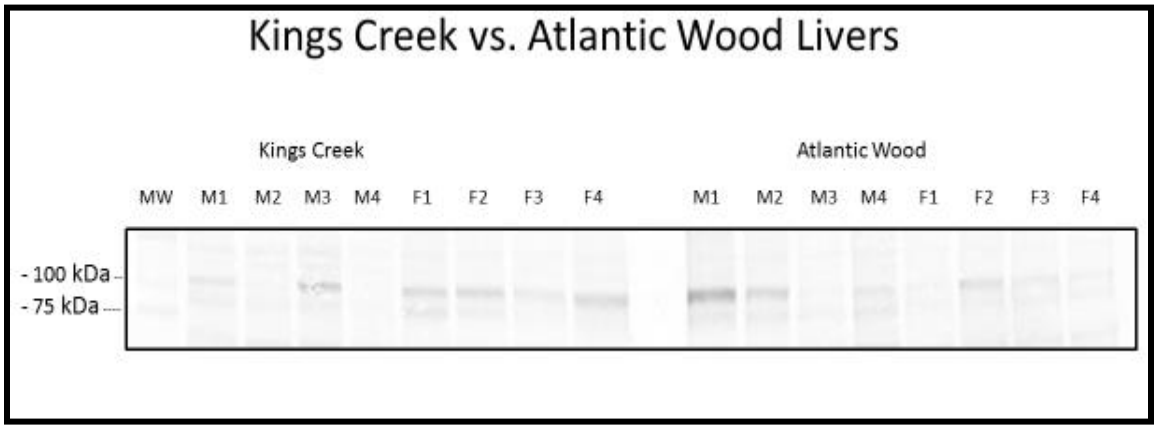


Figure 11. Expression of MVP in Kings Creek vs. Atlantic Wood livers. Eight killifish, 4 males and 4 females, were collected from each site and a western blot was performed using mAb 3F9 for Major Vault Protein.

Figure 12

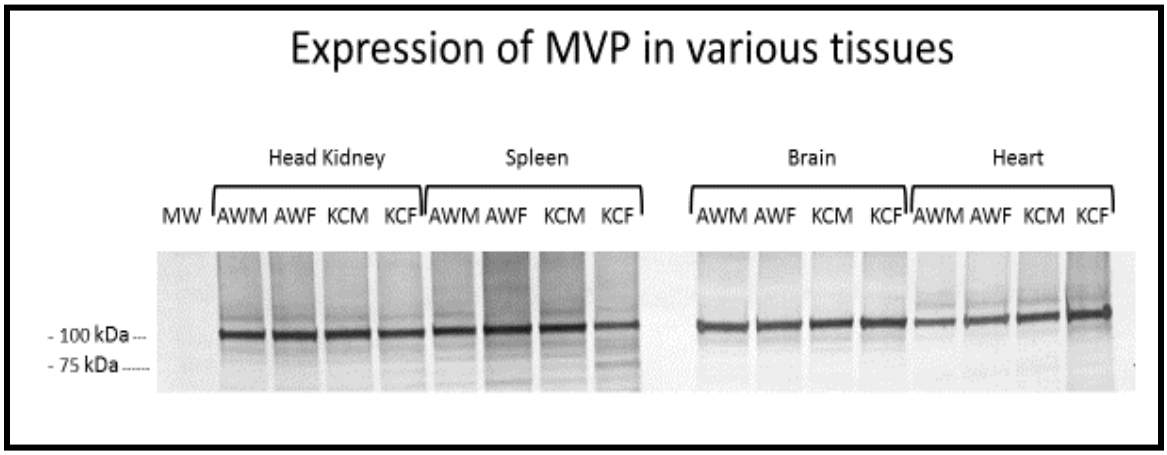


Figure 12. Expression of MVP in various tissues of Atlantic Wood (AW) and Kings Creek (KC) killifish. Male (M) and female (F) organs were harvested from each site, and a western blot was performed using mAb 3F9 for Major Vault Protein.

Figure 13

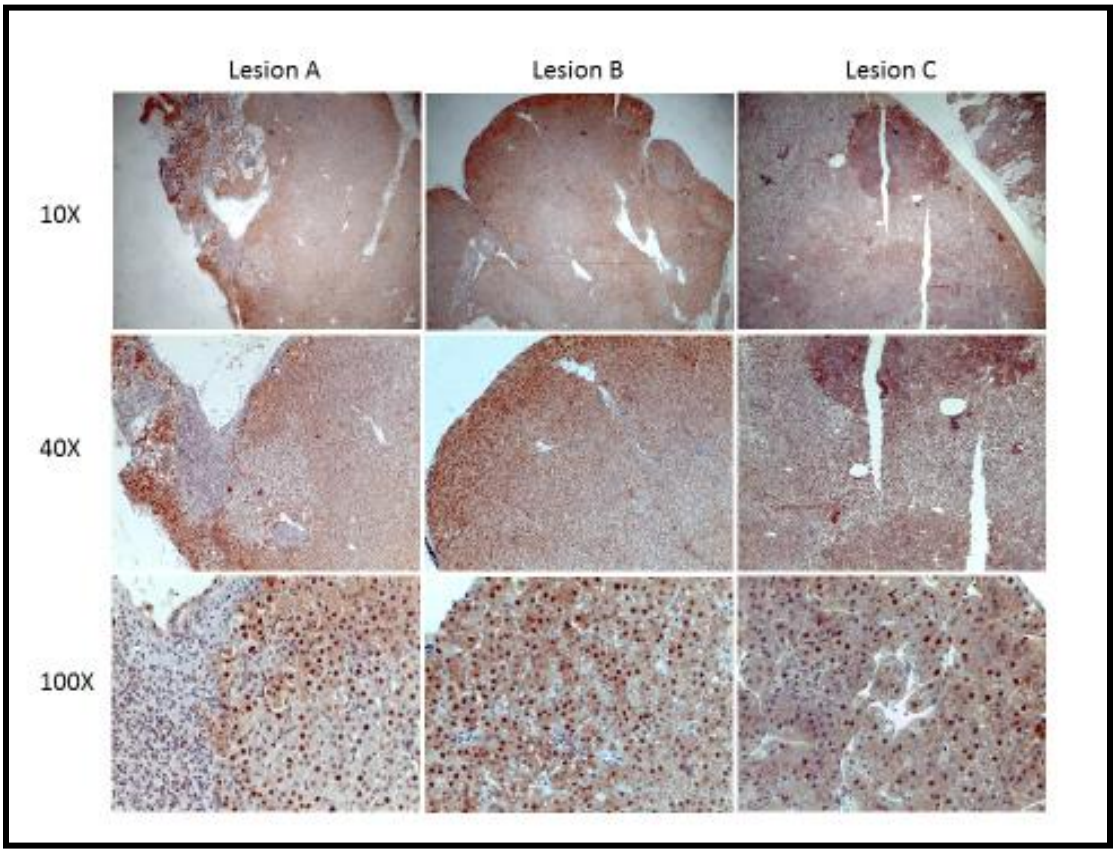


Figure 13. MVP expression in lesions of *Fundulus heteroclitus.* Specimen A, B, and C from Atlantic Wood were immunohistochemically stained with mAb 3F9.

The effects of pharmacological and toxicology treatment on MVP expression in vitro with the channel catfish ovarian cell line CCO.

Changes in MVP expression levels of fish exposed to toxic chemicals were next examined using an *in vitro* model. Channel Catfish Ovarian (CCO) cells were grown in DMEM and in two 24-hour pulsed treatments, the flasks were administered DMSO control or one of the following known cytotoxic treatments: 1.5 µg/ml ethidium bromide, 1 µM retinoic acid, 0.1 µM PMA + $5x10^{-7}$ M A23187, 1 µM benzo-(k)-fluoranthene, or 0.5 µM arsenic. Quantitative PCR was performed for MVP mRNA expression and results showed no significant fold changes between the control or any treatments (Figure 14).

Similarly, CCO cells were grown on glass cover slips in a 6-well plate an administered the same treatment plan described above to examine the subcellular localization of vault particles. These cells were probed for mAb 3F9 and prepared for immunofluorescence. Confocal images of the different cell treatments (Figure 15) show the vaults in various locations. Of particular interest is the extremely low concentration of MVP expression and therefore cellular vaults within the CCO cells treated with 1 µM retinoic acid.

Figure 14

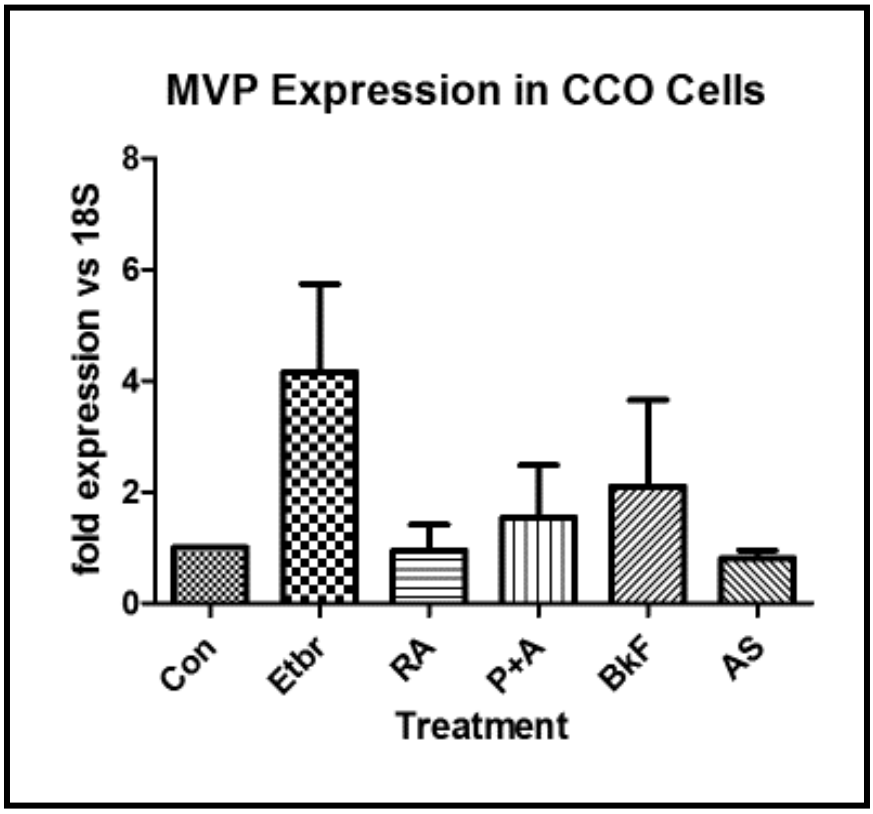


Figure 14. MVP expression in CCO cells treated with DMSO control (Con), 1.5 µg/ml ethidium bromide (EtBr), 1 µM retinoic acid (RA), 0.1 µM PMA + $5x10^{-7}$ M A23187 (P+A), 1 µM benzo-(k)-fluoranthene (BkF), or 0.5 µM arsenic (As). This qPCR data analyzed using the Pfaffl method (Pfaffl, 2001). No significant difference was found.

Figure 15

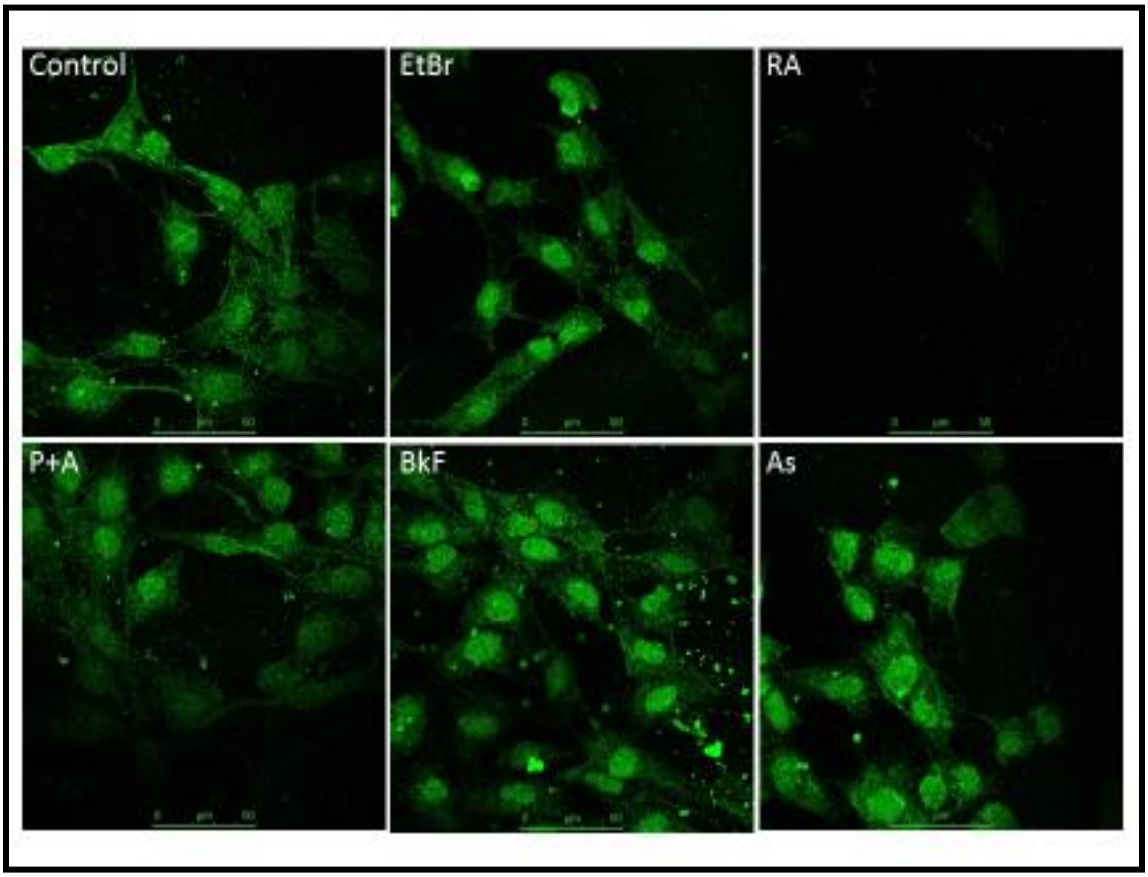


Figure 15. Immunofluorescence of MVP in CCO cells under various treatments: DMSO control, ethidium bromide (EtBr), retinoic acid (RA), PMA and A23187 (P+A), benzo-(k)-fluoranthene (BkF), arsenic (As). Cells were treated with mAb 3F9 and IgG-alexafluor 488 and imaged.

DISCUSSION

In this study, a recombinant channel catfish major vault protein was expressed to then produce a monoclonal antibody (mAb 3F9) for examining the expression of MVP in various fish tissues, and show that MVP (and presumably vault particles) is highly expressed in all tissues examined. Sequence anlysis of the catfish MVP shows that it is highly homologous with MVP in other eukaryotes, and especially rat, electric ray, human, and even a bivalve. As clearly demonstrated, mAb 3F9 recognizes MVP in several distantly related fish, as well as rodent, and will be an important reagent going forward. Moreover, this is the first detailed study of MVP expression and localization in an aquatic animal model.

To date, no one has demonstrated a definitive role, or purpose of vault particles, but mounting evidence suggests that vaults are transport particles for shuttling non-coding vRNA from the cytosol to the nucleus, and also may be involved in acquired drug resistance (Gopinath et al., 2010; Scheffer et al., 1995b; Steiner et al., 2006a). Most studies relating the MVP, or vaults, to drug resistance indicate that cells acquire resistance to chemotherapeutic agents through other mechanisms first, such as MDR-1, or MRPs, and then increase expression of MVP. Therefore, increased expression of MVP comes after a long and protracted exposure to the inducing drug, or xenobiotic compound, as can be seen in benzo-a-pyrene exposed KD-3-1 epidermal cells chronically exposed over a long period to time (Cheng et al., 2000).

If one pays close attention to the literature cited in this thesis, it becomes clear that MVP expression has been studied mostly in cells that are adherent, such as cells of

epithelial origin, neuroectodermal origin, and cells of the immune system that have an adherent phenotype, such as macrophages. In this study, MVP expression is very high in the intestines, endothelial cells lining liver sinusoids and bile canaliculi, on the edge of white pulp of the spleen, in secretory cells of the kidney, and gill epithelia. This observation suggests that MVP, or vaults in general, indeed may have a role in communicating information (or materials) entering or exiting cells lining barriers of various types. While not examined in this study, others show that vaults move along cytoskeletal microtubles (Eichenmüller et al., 2003; Slesina et al., 2006; Slesina et al., 2005) and fit perfectly in the nuclear pore and are of the same size as the nuclear pore complex (Vollmar et al., 2009). This suggests that perhaps vault may move over relatively long distances between the cell membrane and nucleus in large cells, like epithelial-type cells, along microtubule highways carrying information from the environment to the nucleus, and back again.

As can be seen visually by cytochemistry, vault numbers are high in cells with large cytoplasm space, such as the catfish macrophages, and can be seen along the edge of the cell are attachment, and surrounding the nucleus. In smaller cells, such as the lymphocytes shown near macrophages, vault numbers are lower, and reflect the smaller cytoplasm space common to B and T cells. The availability of CCO cells, which are fibroblast-like cells containing large cytoplasmic spaces, provided an opportunity to visualize vaults under various conditions. As is noted in the results section, CCO cells highly express MVP, and vaults can be seen throughout the cytoplasm, and especially surrounding the nucleus. In an attempt to correlate vault particle location and intensity of staining with MVP mRNA expression, the variablity between replicates was fairly high

(n=3), yet treatment with ethidium bromide, a potent DNA damaging agent, increased express of MVP mRNA and intensity of intracellular staining. One of the more surprising findings was the near-complete shut-down of MVP/vault expression in CCO cells treated with retinoic acid. The dose used in this study (1 μM) is commonly used in studies to induce differentiation of mammalian cells (Breitman et al., 1980), and has been used in prior studies with fish (Baier-Anderson et al., 2000) without significant toxicity.

Retinoic acid is derived from vitamin-A, and is a ligand for the transcription factor retinoic acid receptor (RAR), and has a critical role in development, and the process has been studied in many models, from fish to amphibians, to birds and mammals (Rhinn and Dollé, 2012). It is possible that retinoic acid drives CCO cells, which are immortal and blast-like, to a terminally differentiated state leading to apoptosis, or severe toxicity. However, CCO cells treated with retinoic acid were adherent and retained the physical appearance the same as control-treated cells. Clearly, this effect of retinoic acid on MVP/vault expression needs to be further examined, and perhaps in a variety of cell types, including macrophages, dendritic cells, endothelial cells, and other adhernet lines.

It is well documented that MVP, or it's earlier alternative nomenclature, lung resistance protein (LRP) (Scheffer et al., 1995b), is over expressed in several cancer types, including lung, colon, brain, and hemotopoetic neoplasias (Ikeda et al., 1999; Kitazono et al., 1999a; Lu and Shervington, 2008; Ohno et al., 2001; Schroeijers et al., 2000; Slesina et al., 2005). However, it is surprising that few studies are published on the relationship between MVP/vaults and liver cancer. The liver is key to drug and environmental xenobiotic metabolism, and is in the front line environmental contaminants, several classes of which are potent chemical carcinogens. In the field of

environmental toxicology, the liver is the most commonly investigated organ in terms of target organ toxicity. There a five types of primary liver cancers; hepatocellular carcinoma (hepatocytes), hepatoblastomas (hepatocytes), cholangiocarcinomas (bile duct cells), and vascular sarcomas (vessels of the liver), and each have been reported in various models from fish to rats exposed to chemical carcinogenesis (Bunton, 1996; Cohen and Arnold, 2011; Wogan et al., 2004).

One of the most notorious studies of liver cancers due to exposure to envioronmental carcinogens has been carried out for over 20 years at the Atlantic Wood US-EPA Superfund site on the Southern branch of the Elizabeth River near Portsmouth, VA. This site was contaminated for over 70 years with creosote from a wood-treating plant, called Atlantic Wood [see recent indepth review: (Di Giulio and Clark, 2015)]. Creoste is a very toxic and carcinogenic mixture of polyaromatic hydrocarbons also containing traces of metals and other wood-treating preservatives. The local population of killish inhabiting the small lagoon associated with creosote contamination has developed a resistance and tolerance phenotype to the high PAHs at the site (Wills et al., 2010), but as a consequence nearly all adults between two and three years of age have severe liver lesions ranging from altered foci to one of the above liver tumor types (Vogelbein et al., 1999; Vogelbein et al., 1990; Vogelbein and Unger, 2006). An upregulation of multi-drug resistance proteins has been documented in these lesions and healthy tissues surrouding lesions (Cooper, 1999), but MVP/vaults have never been examined in this population. The MVP reagents of this study provided an opportunity to be the first to examine MVP and therefore vaults in tumors from killifish at the AW site.

Upon examination of MVP protein expression in livers and other tissues from killfish, it was clear that MVP expression is highly variable in both populations (Atlantic Wood site vs King's Creek reference site), but there seemed to some livers in the adpated population that expressed more. Other tissues, including heart, brain, spleen, and anterior kidney, did not differ in expression profiles. However, in the liver lesions of killifish from the adapted population MVP expression was very high in some lesions, and just as important, the nucleus of cells in some lesions were brigthly staining, indicating a nuclear location of vaults. Heptoblastoma/hepatocellular carcinomas highly express MVP, and these observations collectively indicate a role in eitiher the progressin of carcinogeneis, or a role in the adpatation of these fish to harsh environmental chemicals. Clearly, this population of killfish offers a great opportunity to explore not only the development of lesions between hatching and adulthood, but also the expression of MVP and vaults over the life span of these fish.

Overall, this study provides the first examination of the egnimatic vault and the MVP in an aquatic animal model, and especially in terms of species cross-reactivity of reagents, tissue and intracelluar localization of MVP, and a first glimpse into the effects of EtBr and retinoic acid on vault/MPV expression. It is my closing thought that following MVP expression in future toxicology and immunotoxicology studies will be as critical to understanding mechanisms of action as are other typical endpoints, such as phase I, II, and III drug metabolizing components, immune function assays, and disease challenges. It is also my hope that this study will add to small, but growing knowledge base on the subject of cellular vaults and MVP.

CHAPTER THREE

CONCLUSION AND FUTURE DIRECTIONS

To my knowledge, this is the first study to develop a monoclonal antibody against a Teleostean MVP, and to examine the expression of MVP protein in various cells and tissues of channel catfish, rainbow trout, and Atlantic killifish. This work clearly demonstrates that MVP, and thus vaults, is highly expressed in epithelial tissues facing the gut lumen and in various vascular tissues, including liver sinusoids, and sinusoids of the spleen. MVP is also expressed in macrophages and other large cells with a high cytoplasmic-nuclear ratio. This is also the first study to examine MVP, also known as the lung resistance protein (LRP) associated with resistance to chemotherapeutic agents, in a fish model for adaptation to harsh chemical environments. Livers from killifish at the Atlantic Wood Superfund site in VA are heavily damaged, with lesions varying from altered hepatic foci to full hepatoblastomas and hepatocellular carcinomas, and these lesions express high levels of MVP. My data clearly show that MVP is localized to the nucleus, or at least nuclear-pore-complex in neoplastic lesions of these fish. Therefore, this work is the first to associate MVP/LRP with a resistance phenotype, or adaptation, to chemicals in the environment. I am very pleased to provide to the scientific community using aquatic animal models for human diseases a high quality reagent for exploring the role of MVP and/or cellular vaults in their particular model.

To improve upon the developing field of cellular vault particles, I suggest some of the following future studies. Variations in temperature and pH cause cellular vaults to change from closed barrel-like to open flower-like formations. This specific function

should be further analyzed for the multidrug resistance of vaults and potential use of vaults in drug administration. Zebrafish are commonly used aquatic models for development. The repeat of this study in performed embryos and immature zebrafish could provide critical data for the importance of MVP during the growth process.

A study involving the gene knockdown of MVP has recently been performed and the sole effect was increased sensitivity of the vaults to doxorubicin which was to be expected since MVP is a structural protein (Herlevsen et al., 2007). Although they are of a much smaller proportion, further research and potential knockdown of the other dominant proteins, vPARP and TEP1, could result in discovery of the functional role of vaults in all organisms. Additional research is also necessary on why the vault has its own specialized RNA, called vRNA, which differs within species.

The locomotion of cellular vaults should also be further explored. The transient linking between one end of a vault and a microtubule is thought to aid in travel across the cytoplasm, however the exact mechanism that allows this to happen has yet to be discovered. Finally, the similarities of the central plug of the nuclear pore complex (NPC) and cellular vaults are undeniable and functional and developmental relationships between the two should be analyzed.

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